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Assembly and DNA binding of recombinant Ku (p70/p80) autoantigen defined by a novel monoclonal antibody specific for p70/p80 heterodimers

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SUMMARY

The Ku autoantigen is a heterodimer of 70 kDa (p70) and ~80 kDa (p80) subunits that is the DNA-binding component of a DNA-dependent protein kinase (DNA-PK). The 350 kDa (p350) catalytic subunit of DNA-PK phosphorylates Sp-1, Oct-1, p53 and RNA polymerase II in vitro, but the precise cellular role of DNA-PK remains unclear. In the present studies, the assembly of p70/p80 heterodimers and the interaction of Ku with DNA was investigated using recombinant vaccinia viruses directing the synthesis of human p70 (p70-vacc) and p80 (p80-vacc), and monoclonal antibodies (mAbs). Expression of human Ku antigens in rabbit kidney (RK13) cells could be demonstrated by immunofluorescent staining because this cell line contains little endogenous Ku. A novel mAb designated 162 stained the nuclei of RK13 cells coinfected with p70-vacc and p80-vacc, but not cells that were infected with either virus alone, suggesting that it recognized the p70/p80 heterodimer but not monomeric p70 or p80. In agreement with the immunofluorescence data, 162 immunoprecipitated both p70 and p80 from extracts of coinfected cells, but did not immunoprecipitate either subunit by itself from extracts of cells infected with p70-vacc or p80-vacc, respectively. Conversely, the binding of 162 to Ku isolated from human K562 cells stabilized the p70/p80 heterodimer under conditions that normally dissociate p70 from p80. The nuclei of cells infected with p70-vacc alone could be stained with mAb N3H10 (anti-p70) and cells infected with p80-vacc alone could be stained with mAb 111 (anti-p80), indicating that the formation of p70/p80 heterodimers was not required for nuclear transport. Finally, free recombinant and cellular p70 both bound to DNA efficiently in vitro, suggesting that free p70, like the p70/p80 heterodimer, serves as a DNA-binding factor. Moreover, free human p70 could be released from the nuclei of p70-vacc-infected RK13 cells by deoxyribonuclease I treatment, suggesting that it was associated with chromatin in vivo. The nuclear transport of free p70 and the association of free p70 with chromatin in vivo raise the possibility that newly synthesized cellular p70 might undergo nuclear transport and DNA-binding prior to dimerization with p80 or assembly with p350.

Key words: Ku autoantigen, recombinant vaccinia virus, DNA-dependent protein kinase, DNA-binding protein

INTRODUCTION

The Ku antigen is a DNA-binding factor recognized by autoantibodies from the sera of certain patients with scleroderma-polymyositis overlap syndrome, systemic lupus erythematosus (SLE), and related disorders (Mimori et al., 1981; Reeves, 1985; Yaneva and Busch, 1986), which is closely related or identical to the DNA-binding factors NFIV (Stuiver et al., 1990), PSE1 (Knuth et al., 1990), TREF (Roberts et al., 1989), EPB-80 (Falzon et al., 1993) and E1BF (Hoff and Jacob, 1993), and to a group of proliferation-sensitive nuclear proteins of 64-82 kDa, identified by isoelectric focusing (Bravo and Celis, 1982; Celis et al., 1987; Stuiver et al., 1991). Ku consists of a heterodimer of 70 kDa (p70) and ~80 kDa (p80) subunits (Mimori et al., 1981; Reeves, 1985; Yaneva et al., 1985) that binds to double-stranded DNA termini, nicks, or single- to double-strand transitions (Mimori and Hardin, 1986; Blier et al., 1993; Falzon et al., 1993). Sequence specific DNA-binding has also been reported (Knuth et al., 1990; Roberts et al., 1989; May et al., 1991). Several studies have suggested that binding to DNA in vitro is mediated largely by p70 (Mimori and Hardin, 1986; Allaway et al., 1989; Chou et al., 1992), but other reports suggest that dimerization of p70 with p80 is required for DNA binding (Griffith et al., 1992; Kaczmarski and Khan, 1993). Ku is the DNA-binding component of a DNA-dependent protein kinase (DNA-PK) that phosphorylates

several chromatin-bound proteins in vitro, including the transcription factors Sp-1 and Oct-1, RNA polymerase II and the p53 tumor suppressor protein (Lees-Miller et al., 1990; Dvir et al., 1992; Anderson and Lees-Miller, 1992; Gottlieb and Jackson, 1993). The catalytic activity of DNA-PK is carried by a 350 kDa polypeptide (p350) (Lees-Miller et al., 1990; Carter et al., 1990). Although Ku and DNA-PK have been proposed to serve in transcriptional regulation (Knuth et al., 1990), DNA repair/transposition, or cell cycle regulation (Mimori and Hardin, 1986; Feldmann and Winnacker, 1993; Anderson, 1993), the precise roles in vivo of the p70, p80 and p350 subunits, and the DNA-PK complex, have not been determined. The low level of Ku and DNA-PK activity in certain non-primate cell lines (Bravo and Celis, 1982; Celis et al., 1987; Wang et al., 1993; Lees-Miller et al., 1992; Anderson and Lees-Miller, 1992) suggests that such cell lines may be advantageous for investigating the assembly of recombinant DNA-PK complexes and for examining the regulation of DNA-PK activity. As a step toward defining the role of Ku in DNA-PK function, we have assembled human p70/p80 heterodimers in rabbit kidney (RK13) cells using recombinant vaccinia viruses, and have identified a mAb specific for the p70/p80 heterodimer. Expression of p70 using the vaccinia virus system has also made it possible to show that free p70 serves as a DNA-binding factor, suggesting that the DNA-PK complex may be assembled on chromatin.

MATERIALS AND METHODS

Assembly of full-length p70 and p80 cDNAs

The large *Eco*RI fragment of p70 clone 70.5 (nucleotides 379-2021) (Reeves and Sthoeger, 1989) was inserted in pBKS− (Stratagene, La Jolla, CA) downstream of the T7 RNA polymerase promoter. Plasmid pBKS-70.5 was partially digested with *Eco*RI, and the small *Eco*RI fragment of p70 clone 70.30 (nucleotides 1-378) (Reeves and Sthoeger, 1989) was inserted. A recombinant with the complete coding sequence plus the 5′and 3′ untranslated regions of the p70 cDNA (pBKS-70) was identified by restriction endonuclease mapping and confirmed by DNA sequencing. In vitro transcription of pBKS-70 using T7 RNA polymerase (Sambrook et al., 1989) followed by in vitro translation in a reticulocyte lysate system (Promega, Madison, WI) resulted in synthesis of an immunoreactive 70 kDa protein with mobility identical to that of human cellular p70. Similarly, a 2.6 kb *Eco*RI fragment containing the complete coding sequence and 5′ and 3′ untranslated regions of p80 (clone 20a; Reeves et al., 1991) was subcloned into the *Eco*RI site of pBKS− downstream of the T7 promoter to form plasmid pBKS-80. In vitro transcription/translation resulted in the production of an immunoreactive ~80 kDa protein with mobility identical to that of human cellular p80.

Recombinant vaccinia viruses

The insert of clone pBKS-70 was excised with *Bam*HI and *Sal*I and inserted into the vaccinia recombination vector pgpt-ATA18 to generate plasmid pgptATA18-70. The vector pgptATA18 was derived from pATA18 (Stunnenberg et al., 1988) by inserting an I3gpt cassette into the *Cla*I site upstream of the 11K late promoter sequence of pATA18 (Stunnenberg et al., 1988). The early/intermediate I3 promoter drives the *Escherichia coli* xanthine guanine phosphoribosyl transferase (gpt) gene for selection purposes (Falkner and Moss, 1988). Similarly, the insert of pBKS-80 was excised and inserted into the *Eco*RI site of pgptATA18 to generate plasmid pgptATA18-80. Human 143 tk− cells were infected with wild-type vaccinia virus at a m.o.i. of 0.1, and transfected 2 hours later by 1 μg of supercoiled plasmid (pgptATA18-70 or pgptATA18-80) coprecipitated with calcium phosphate (Sambrook et al., 1989). Recombinants p70-vacc and p80-vacc were selected by growth in semisolid medium containing 250 μg/ml xanthine, 15 μg/ml hypoxanthine and 25 μg/ml mycophenolic acid on RK13 cells. Individual plaques were amplified on 143 tk− cells in the presence of 100 μg/ml 5-bromodeoxyuridine and further amplified on RK13 cells. Recombinant p70 or p80 proteins were identified in cell lysates by SDS-PAGE and immunoblotting using human autoimmune serum and specific mAbs.

Cell culture and virus infection

Rabbit kidney (RK13) cells (from the American Type Culture Collection, Rockville, MD) were grown in Eagle's modified essential medium (EMEM) containing non-essential amino acids, penicillin/streptomycin, and 10% fetal bovine serum. Confluent cultures in 100 mm tissue culture plates were washed twice with phosphate buffered saline (PBS), before infecting at 37°C with vaccinia virus at a m.o.i. of 3 in 1.0 ml of serum-free EMEM. In some experiments, the cells were coinfected with two recombinant vaccinia viruses, each at a m.o.i. of 3. After 1 hour, 9 ml of complete medium containing 5% fetal bovine serum was added, and the cells were grown in a 5% CO_2 atmosphere for an additional 12-22 hours at 37°C before harvesting.

Monoclonal antibodies

Murine mAbs specific for the human Ku antigen have been characterized (Reeves, 1985; Knuth et al., 1990; Wang et al., 1993). The isotypes and specificities of the mAbs were as follows: 162, IgG2a that immunoprecipitates the p70/p80 dimer, but is unreactive by western blotting; 111, IgG1 specific for human p80 (amino acids 610-705); S10B1, IgG1 reactive with human p80 (amino acids 8-221) with weaker binding to p70; N9C1, IgG1 reactive with p80 (amino acids 1-374) by western blot; N3H10, IgG2b reactive with human p70 (amino acids 506-541), and S5C11, IgG1 reactive with p70.

Indirect immunofluorescence

RK13 cells grown on coverslips for 12-18 hours after vaccinia virus infection were fixed in methanol for 10 minutes at –20°C, followed by blocking for 1 hour at 22°C in PBS containing 10% bovine calf serum. The cells were incubated with anti-Ku mAbs (1:50 dilution of ascitic fluid in PBS/10% fetal bovine calf serum for 30 minutes at 22°C), washed with PBS, and then incubated for 30 minutes with 1:50 FITC-conjugated goat anti-mouse immunoglobulin antibodies (Tago, Burlingame, CA). The cells were washed and counterstained with Evans blue (0.0005% in PBS) before photographing.

Immunoblotting

Immunoblot analysis of the recombinant p70 and p80 proteins from RK13 cells and Ku antigens from uninfected human K562 (erythroleukemia) cells was performed using human autoantibodies or murine mAbs as described (Reeves et al., 1991; Wang et al., 1993). Ascitic fluid from mAbs N3H10, S10B1 and 111 was diluted 1:500 for probing immunoblots, followed by 1:1500 alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Tago).

Immunoprecipitation

RK13 cells were infected with recombinant vaccinia viruses or wild-type vaccinia virus as described above, except that the culture medium was methionine-free RPMI 1640 containing 5% dialyzed fetal bovine serum, 3% RPMI 1640 (with methionine), and antibiotics. Three hours after infection, [^{35}S]methionine/cysteine (Translabel, ICN Biomedicals, Inc., Costa Mesa, CA; 25 μCi/ml) was added to the culture medium, and the cells were labeled for 17 hours before harvesting. The cells were washed once with PBS, collected by centrifugation, resuspended at 5×10^7/ml in ice-cold KM buffer (10 mM MES, pH 7.2, 10 mM NaCl, 1.5 mM $MgCl_2$) containing 1% Triton X-100, 0.5

mM phenylmethylsulfonyl fluoride (PMSF), and aprotinin (0.3 trypsin inhibitor unit/ml; from Sigma Chemical Company, St Louis, MO). After incubating for 10 minutes on ice, the cells were vortexed for 1 minute, NaCl was added to a final concentration of 0.15 M, and the cells were incubated for an additional 20 minutes on ice. The lysate was cleared by centrifugation (10,000 ***g*** for 10 minutes) and incubated for 3 hours with mAb-coated Protein A Sepharose beads at 4°C (Chou et al., 1992). The beads were then washed twice with mixed micelle buffer (MMB: 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.25 M sucrose, 0.5% SDS, 2.5% Triton X-100), and three times with NET buffer (0.15 M NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA). Immunoprecipitated proteins were eluted from the beads by boiling in SDS sample buffer, and analyzed by SDS-PAGE (Reeves, 1985).

Immunoprecipitation with dissociation of p70/p80

K562 cells radiolabeled with ^{35}S as above were sonicated at a concentration of 10^7/ml in NET buffer containing 0.3% Nonidet P-40 (NET-NP40) and cleared twice for 15 minutes by centrifugation at 10,000 ***g***. Cell lysate from 2×10^6 cells was immunoprecipitated with 3 μl of ascitic fluid containing anti-Ku mAbs. For immunoprecipitation with mouse IgG1 mAbs 111, S10B1, S5C11 and N9C1, 12 μl of rabbit anti-mouse IgG (1 mg/ml) was also added to the beads. The beads were then washed by one of two protocols: (1) three washes with MMB followed by one wash with NET buffer; or (2) two washes with NET buffer containing 1.5 M NaCl and 0.3% NP40 (1.5 M NaCl NET-NP40), two washes with MMB, and one NET wash. The immunoprecipitated proteins were eluted by boiling in SDS sample buffer, analyzed on 10% SDS-polyacrylamide gels, and fluorographed. In some experiments, beads were washed with NET-NP40 buffer containing 0.15, 0.5, 1.0 or 1.5 M NaCl, followed by three washes with MMB and one with NET. In other experiments, the beads were washed with 1.5 M NaCl NET-NP40 buffer alone, MMB followed by 1.5 M NaCl NET-NP40 buffer, or 1.5 M NaCl, NET-NP40 followed by MMB.

Effect of DNA on stability of Ku

The possibility that DNA might protect p70/p80 from dissociation was studied by treating immunoprecipitates with deoxyribonuclease I (DNase) then washing with MMB. ^{35}S-labeled K562 cell extract was immunoprecipitated with 162 and N3H10 as above. Immunoprecipitates were then incubated for 30 minutes on ice with either 200 μl of 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.3 % NP40, 2 mM $MgCl_2$, or the same buffer containing DNase (type II, from Sigma Chemical Company, St Louis, MO) at 50 μg/ml. The beads were washed with MMB and NET, analyzed by SDS-PAGE, and autoradiographed as above. In other experiments, ^{35}S-labeled K562 cell extract was immunoprecipitated with 162 and N3H10 as before. After washing the beads three times with 1.5 M NaCl, NET-NP40 buffer, and once with NET, 100 μl of sonicated salmon sperm DNA in NET (1 mg/ml from Sigma) or NET alone was added and incubated for 1 hour at 22°C. Beads were then washed with MMB and NET and analyzed as above.

DNA binding of Ku antigens

p70/p80 dimers and free cellular p70 purified on mAb N3H10 or 162-coated Protein A Sepharose beads were tested for binding to ^{32}P-labeled bacteriophage lambda *Hin*dIII fragments in a DNA immunoprecipitation assay as described (Chou et al., 1992). Briefly, linear double-stranded DNA fragments generated by *Hin*dIII digestion of bacteriophage lambda DNA (New England Biolabs, Beverly, MA) were end-labeled with [^{32}P]dATP (3,000 Ci/mmol, DuPont-New England Nuclear, Boston, MA) using Klenow fragment (Boehringer Mannheim Corporation, Indianapolis, IN) as described (Ausubel et al., 1993). K562 cells were sonicated in NET-NP40 buffer at 5×10^7/ml and the extract was cleared by centrifuging twice at 10,000 ***g*** for 15 minutes. Cell extract from 10^7 cells was immunoprecipitated with 3 μl of 162 or N3H10 ascites. Beads were washed with 0.15 M NaCl NET-NP40 buffer, 1.5 M NaCl NET-NP40 buffer, 1.5 M NaCl NET-NP40 buffer followed by MMB, or 1.5 M NaCl NET-NP40 buffer followed sequentially by MMB and 1.5 M NaCl NET-NP40 buffer. The beads were then incubated with ~50 ng labeled DNA and washed again. Labeled DNA bound to the affinity-purified p70/p80 or p70 antigens was recovered by digesting the beads with proteinase K, phenol extraction and ethanol precipitation, then analyzed by agarose gel electropheresis and autoradiography. Ku antigens expressed in recombinant vaccinia virus-infected RK13 cells were tested for DNA binding in the same manner except that the beads were prewashed with buffer containing 0.5 M NaCl to remove endogenous DNA.

Release of Ku from nuclei by DNase

RK13 cells were harvested 22 hours after infection with vaccinia virus and washed once with PBS. The cell pellet was resuspended and incubated for 30 minutes at 10^8 cells/ml in ice-cold KM buffer containing 0.5% Triton X-100, 1 mM dithiothreitol, 1 μg/ml leupeptin, aprotinin and PMSF. The nuclei were pelleted by centrifugation (800 ***g*** for 10 minutes), and the supernatant was saved for SDS-PAGE analysis. The nuclei were then washed twice in KM, and resuspended at 10^8/ml in the same buffer without Triton X-100 in the presence or absence of 50 μg/ml DNase (Sigma) for 30 minutes on ice. EGTA was then added to a final concentration of 5 mM, the nuclei were pelleted by centrifugation, and the supernatant was collected. Proteins released by DNase were precipitated with 90% ethanol, resuspended in SDS sample buffer, and analyzed by gel electrophoresis and immunoblotting. Uninfected human cells (K562 erythroleukemia cell line) were extracted similarly and analyzed by SDS-PAGE and immunoblotting.

RESULTS

We have shown previously that mAbs specific for Ku are only weakly reactive with non-primate cells (Wang et al., 1993). In the case of mAbs 111 and S10B1, the weak reactivity reflects specificity for the human p80 protein. In the case of N3H10 and 162, which crossreact with murine Ku, the weak reactivity is due to the extremely low levels of Ku in non-primate compared with primate cells. The low level of Ku in non-primate cells makes cell lines such as RK13 suitable for detecting the expression of recombinant human Ku proteins.

Expression of recombinant human Ku proteins

RK13 cells were infected for 22 hours with p70-vacc, p80-vacc, both viruses or wild-type (wt) vaccinia virus, and Ku expression was analyzed by immunoblotting. mAb N3H10 (anti-p70) recognized a 70 kDa protein from cells infected with p70-vacc or coinfected with p70-vacc plus p80-vacc (Fig. 1, left panel). This protein displayed identical electrophoretic mobility to that of the authentic human p70 protein from uninfected human K562 cells (Fig. 1, lane K), and was absent in lysates of cells infected with p80-vacc or wild-type vaccinia virus. Weak binding of N3H10 to the rabbit p70 protein was also detectable (see p80-vacc and wt lanes), consistent with the previously reported crossreactivity of N3H10 with non-primate p70 (Wang et al., 1993). Estimation of the relative amounts of p70 and p80 by densitometry suggested that uninfected human cell lines contain 40- to 60-fold more Ku than rodent cell lines (data not shown).

S10B1 (anti-p80 plus p70) (Knuth et al., 1990; Wang et al., 1993) recognized prominent ~80 and 70 kDa proteins in

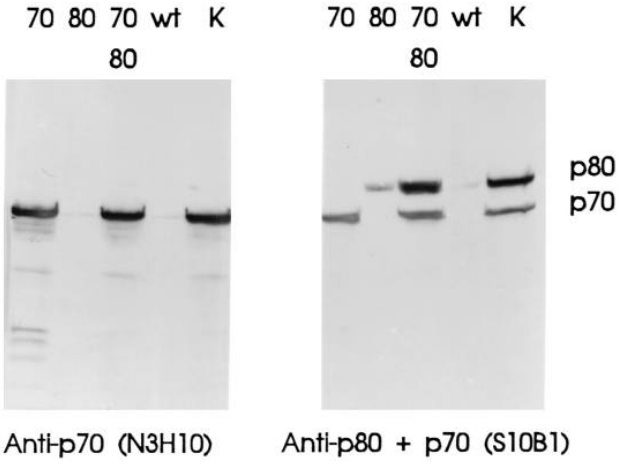


Fig. 1. Immunoblot analysis of recombinant p70 and p80. RK13 cells were infected with p70-vacc (70), p80-vacc (80), both viruses (70/80), or wild-type vaccinia virus (wt). Vaccinia virus-infected RK13 cells and uninfected human K562 cells (K) were solubilized in SDS-sample buffer, and subjected to SDS-PAGE and immunoblot analysis using mAb N3H10 (anti-p70) or S10B1 (anti-human p80, weakly crossreacting with p70).

extracts of recombinant vaccinia virus-infected cells (Fig. 1, right panel). Only the 70 kDa or the 80 kDa protein was detected in lysates from cells infected with p70-vacc or p80-vacc, respectively, whereas both proteins were detected in lysates of cells coinfected with p70-vacc and p80-vacc. The recombinant ~80 kDa and 70 kDa proteins comigrated on SDS gels with authentic human p80 and p70 (lane K). Considerably less p80 was detected in lysates of cells infected with p80-vacc compared with cells coinfected with p80-vacc plus p70-vacc, suggesting that the p80 protein was expressed poorly in cells infected with p80-vacc, but not in cells coinfected with p70-vacc plus p80-vacc. Weak cross-reactivity of S10B1 with an endogenous rabbit protein migrating slightly ahead of human p80 was also apparent (see wt lane). These results suggest that high levels of immunoreactive human p70 and p80 of appropriate electrophoretic mobilities were expressed in the infected RK13 cells.

Subcellular localization of recombinant Ku proteins

The subcellular distribution of human Ku antigens synthesized in vaccinia-infected RK13 cells was investigated by indirect immunofluorescence using mAbs 111 (anti-p80), N3H10 (anti-p70), and 162 (specific for an SDS-sensitive conformational determinant of Ku). At 12 hours after infection, 162 stained the nuclei of RK13 cells coinfected with p70-vacc and p80-vacc, but not cells infected with either p70-vacc or p80-vacc alone, or wild-type (wt) vaccinia virus (Fig. 2, top row), suggesting that it recognized an antigenic determinant that depends on p70/p80 dimerization. mAb 111 stained the nuclei of coinfected cells, and stained weakly the nuclei of cells infected with

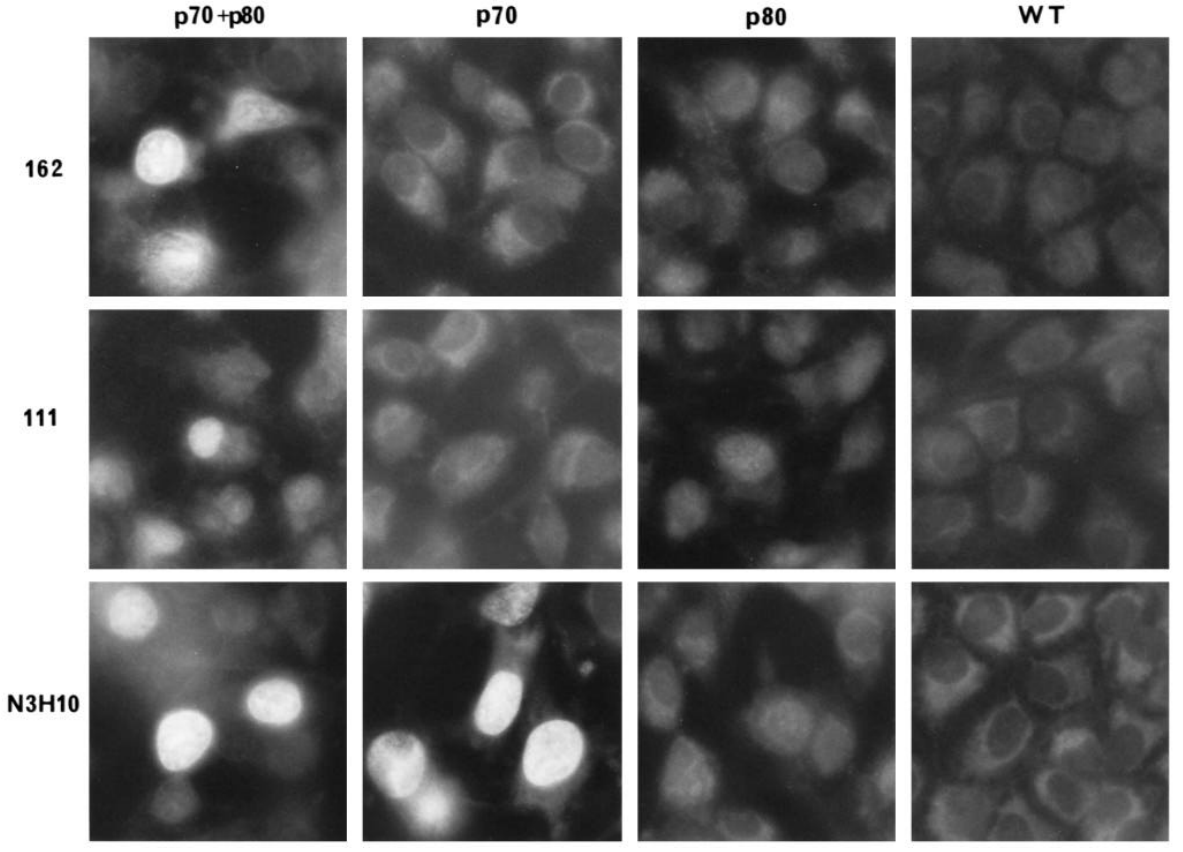


Fig. 2. Immunofluorescence of RK13 cells infected for 12 hours with recombinant vaccinia virus. RK13 cells were infected with p70-vacc (p70), p80-vacc (p80), both viruses (p70+p80), or wild-type vaccinia virus (WT). At 12 hours after infection, the cells were fixed and stained with mAb 162, 111 or N3H10, followed by FITC-conjugated goat anti-mouse IgG antibodies.

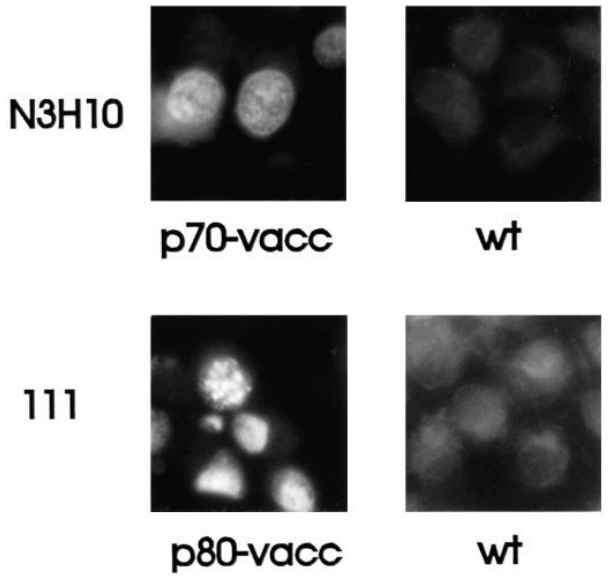


Fig. 3. Immunofluorescence of RK13 cells infected for 18 hours with recombinant vaccinia virus. RK13 cells were infected with p70-vacc, p80-vacc or wild-type vaccinia virus (wt). At 18 hours after infection, the cells were stained with mAb N3H10 or 111, followed by FITC-conjugated goat anti-mouse IgG antibodies.

p80-vacc alone, but did not stain cells infected with p70-vacc or wild-type vaccinia virus (middle row). N3H10 stained the nuclei of coinfected cells and cells infected with p70-vacc alone, but not cells infected with p80-vacc or wild-type virus (bottom row). The subcellular distribution of p70 (detected by N3H10) and p80 (detected by 111) was predominantly or exclusively nuclear in singly infected cells, suggesting that the recombinant human p70 and p80 proteins were transported to the nucleus, and that formation of a human p70/human p80 dimer was not necessary for nuclear localization. Nucleolar staining was also visible in cells infected with p70-vacc or coinfected with p70-vacc and p80-vacc.

Because of the weak staining displayed by the anti-p80 mAb (111) 12 hours after infection, immunofluorescence was also performed 18 hours after infection (Fig. 3). Although the cell morphology was somewhat more distorted than at 12 hours, nuclear staining of p80-vacc-infected cells by 111 was clearly visible at 18 hours after infection. The human p80 protein was distributed in large aggregates within the nuclei of infected RK13 cells in a very different pattern from that of the Ku in uninfected human cells (Reeves, 1985) or p70/p80 coinfected cells. In contrast, the distribution of p70 at 18 hours after infection closely resembled that of the p70/p80 dimer in uninfected cells (Reeves, 1985) and in RK13 cells coinfected with p70-vacc and p80-vacc (Fig. 2, 162 mAb). The immunofluorescence studies indicated that the human Ku proteins were localized to the nuclei of RK13 cells infected with the recombinant vaccinia viruses, and that p70 and the p70/p80 dimer may associate with similar or identical sites within the nucleus. In contrast, the intranuclear distribution of p80 was markedly different than that of p70 or the p70/p80 dimer.

Dimerization of p70 and p80 in infected cells

The staining pattern of 162 suggested that this mAb might specifically recognize the p70/p80 dimer (Fig. 2), an interpretation that was also supported by immunoprecipitation studies (Fig. 4). mAb N3H10 (anti-p70) immunoprecipitated the human p70 protein from lysates of RK13 cells infected with p70-vacc, and both p70 and p80 from lysates of coinfected cells, but did not immunoprecipitate human p80 from lysates of cells infected with p80-vacc (Fig. 4, left). N3H10 also immunoprecipitated a more weakly labeled band migrating at 36 kDa from lysates of coinfected cells (arrow). In contrast, mAb 111 (anti-p80), immunoprecipitated p80 from lysates of RK13 cells infected with p80-vacc, and both p70 and p80 from coinfected cells, but did not immunoprecipitate p70 from lysates of cells infected with p70-vacc (Fig. 4, center). The 36 kDa band was also faintly visible in 111 immunoprecipitates after longer exposures (not shown). mAb 162 immunoprecipitated both p70 and p80 from lysates of coinfected RK13 cells, but did not immunoprecipitate either p70 or p80 from lysates of RK13 cells infected with p70-vacc or p80-vacc alone (Fig. 4, right). mAb 162 crossreacted with

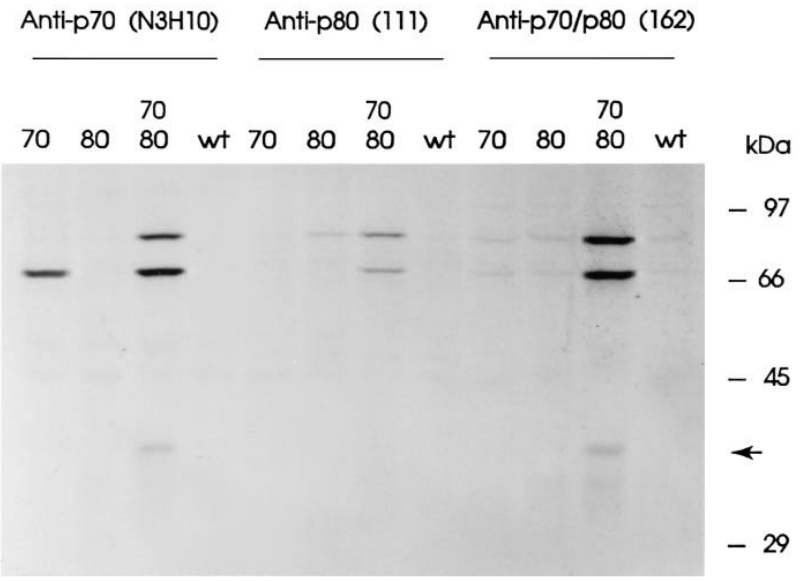


Fig. 4. Immunoprecipitation of recombinant Ku proteins. RK13 cells were infected with p70-vacc (70), p80-vacc (80), both viruses (70/80) or wild-type vaccinia virus (wt) and labeled with [^{35}S]methionine/cysteine. Radiolabeled cells were harvested 22 hours after infection, and lysates were immunoprecipitated with mAb N3H10 (anti-p70), 111 (anti-p80) or 162 (anti-p70/p80), followed by SDS-PAGE and autoradiography. Molecular mass standards (in kDa) are indicated on the right. The position of a 36 kDa band, visible in immunoprecipitates of coinfected cell lysates with all three mAbs, is indicated by arrow.

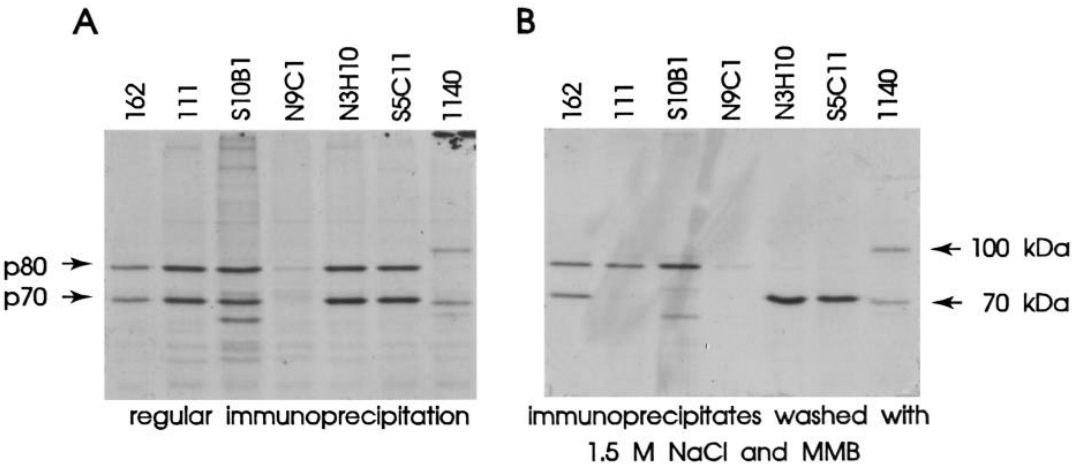


Fig. 5. Dissociation of the p70/p80 heterodimer. K562 cells were labeled with [^{35}S]methionine/cysteine, and extracts were immunoprecipitated onto Protein A Sepharose beads using mAbs 162 (anti-p70/p80 dimer), 111, S10B1, N9C1 (anti-p80) or N3H10, S5C11 (anti-p70). Immunoprecipitation with mouse antiserum 1140, which immunoprecipitates unrelated proteins of 100 and 70 kDa, is shown for comparison. (A) Immunoprecipitates washed with MMB followed by NET. All anti-Ku mAbs except N9C1 immunoprecipitated both p70 and p80. N9C1 immunoprecipitated only a weak p80 band. (B) Immunoprecipitates washed with 1.5 M NaCl, NET-NP40 followed by MMB. Note the different immunoprecipitation patterns compared with (A). p70, p80 or both, were immunoprecipitated according to the specificity of the mAbs under these conditions. S10B1 immunoprecipitated p80 as well as the previously described crossreactive 63 kDa protein in dissociating conditions.

the endogenous rabbit Ku antigen, consistent with previous observations (Wang et al., 1993) and, like N3H10 and 111, immunoprecipitated the 36 kDa band from lysates of coinfected cells (arrow). N3H10 was reactive on immunoblots with the 36 kDa polypeptide immunoprecipitated by 162, suggesting that it was derived from p70 by proteolytic cleavage (data not shown). The co-immunoprecipitation of p70 and p80 by mAbs specific for p80 and p70, respectively, and the immunoprecipitation of p70 and p80 by mAb 162 from lysates of RK13 cells infected with both p70-vacc and p80-vacc, strongly suggest that p70/p80 heterodimers were assembled in coinfected cells, and that 162 recognizes the heterodimer, as suggested by the immunofluorescence data. The specificity of 162 for p70/p80 dimers was investigated further by subjecting affinity-purified p70/p80 heterodimers to high salt and/or detergent treatment (Figs 5-6).

mAb 162 protects p70/p80 heterodimer from dissociation

Anti-Ku mAbs 162, 111, S10B1, N3H10 and S5C11 all immunoprecipitated both p70 and p80 under standard conditions, as expected (Fig. 5A). An irrelevant murine antiserum (1140) immunoprecipitated proteins of ~70 and 100 kDa as expected, but not the p70 or p80 Ku proteins. A strikingly different pattern was observed if the immunoprecipitates were washed with 1.5 M NaCl NET-NP40 buffer followed by MMB (Fig. 5B). mAbs specific for p80 (111 and S10B1) retained p80 but not p70 on the beads, whereas mAbs specific for p70 (N3H10 and S5C11) retained p70 but not p80. In contrast, mAb 162 retained both p70 and p80, consistent with its recognition of an epitope composed of p70 bound to p80. Moreover, 162 appeared to protect p70/p80 heterodimers from dissociation during high salt and MMB washing. N9C1 was unusual because although strongly reactive with p80 by western blotting (Wang et al., 1993), it immunoprecipitated p80 weakly, but not p70, under standard conditions (Fig. 5A). This pattern was unaffected by washing with 1.5 M NaCl plus MMB (Fig. 5B), and was also apparent in immunoprecipitates washed only with NET-NP40 (not shown), suggesting that N9C1 recognizes an epitope of p80 that is inaccessible after dimerization of p80 with p70.

The effect of salt concentration on p70/p80 dissociation is shown in Fig. 6. The immunoprecipitation pattern displayed by mAb 162 was unaffected by washing with NaCl at concentrations ranging from 0.15 to 1.5 M before MMB washing (Fig. 6A). In contrast, although both p70 and p80 were visible in N3H10 immunoprecipitates washed sequentially with NET (0.15 M NaCl) and MMB, washing with 0.5, 1.0 or 1.5 M NaCl followed by MMB released p80 from N3H10 immunoprecipitates (Fig. 6A, right). The critical concentration of NaCl for releasing p80 from p70 when followed by MMB washing was approximately 0.3 M (data not shown). As shown in Fig. 6B, N3H10 retained both p70 and p80 if the beads were washed with 1.5 M NaCl NET or MMB alone, whereas only p70 was retained if the beads were washed sequentially with 1.5 M NaCl NET and MMB (Fig. 6B, left), suggesting that high ionic strength plus MMB was necessary to dissociate Ku. The order of washing (high salt followed by MMB vs MMB followed by high salt) appeared to be important. Dissociation occurred only in immunoprecipitates washed first with 1.5 M NaCl and then with MMB; surprisingly, the reverse order (MMB followed by 1.5 M NaCl) did not dissociate the complex (not shown). Human autoimmune serum from patient JM, containing autoantibodies to both p70 and p80, retained both proteins on the beads, as expected (Fig. 6B, right).

To examine the effect of DNA binding on the stability of the p70/p80 dimer, 162 and N3H10 immunoprecipitates were treated with DNase before washing with high salt and/or MMB. DNase treatment had little or no effect on the immunoprecipitation pattern (data not shown), suggesting that DNA

binding had little effect on the stability of p70/p80 dimers. Conversely, adding back DNA to the p70/p80 dimer after 1.5 M NaCl treatment (which dissociates Ku from DNA; Reeves, 1987), did not prevent dissociation of the dimer by subsequent treatment with MMB (not shown).

Free p70 binds DNA in vitro

We have shown previously that fusion proteins carrying amino acids 536-609 of p70 can bind DNA in vitro (Chou et al., 1992). However, in vitro translated full-length p70 did not bind to DNA in other studies (Griffith et al., 1992). To investigate further the DNA-binding properties of p70, cellular p70 and recombinant p70 from p70-vacc-infected cells were affinity-purified and tested for DNA binding. To examine whether the cellular p70 protein from human K562 cells can bind DNA, p70/p80 heterodimers affinity-purified on N3H10-Protein A Sepharose beads were dissociated by high salt and detergent treatment. Complete dissociation of p70/p80 on the beads was ascertained by SDS-PAGE as shown in Fig. 5. The affinity-purified cellular p70 was then tested for binding to linear DNA fragments in vitro (Fig. 7A).

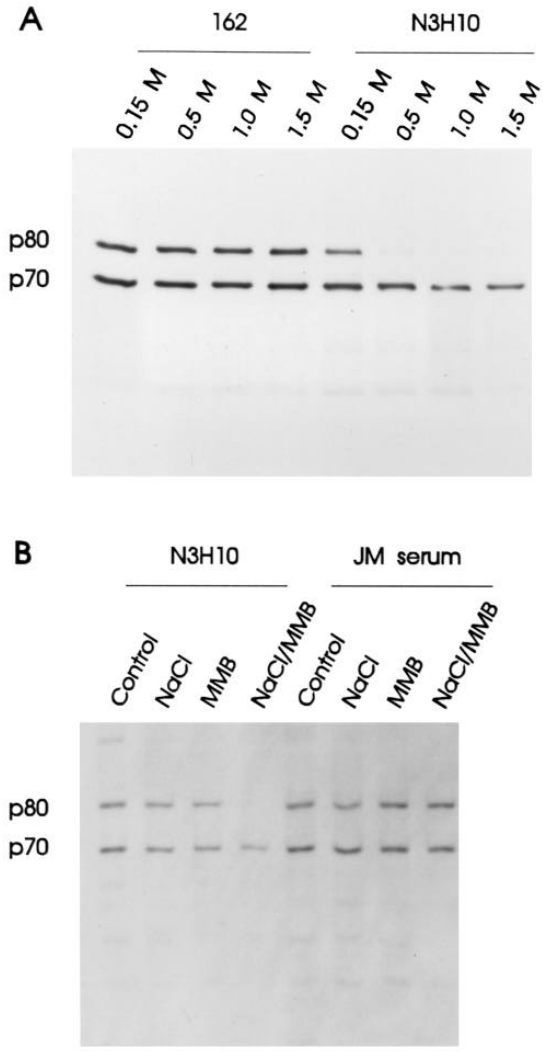


Fig. 6. Conditions for dissociating Ku heterodimer. K562 cells were labeled with [^{35}S]methionine and cysteine, and extracts were immunoprecipitated onto Protein A Sepharose beads. (A) Effect of high salt plus detergent. Immunoprecipitates using mAbs 162 or N3H10 were washed with buffers containing 0.15, 0.5, 1.0 or 1.5 M NaCl-NP40 followed by MMB, then analyzed by SDS-PAGE and autoradiography. Positions of p70 and p80 are indicated. (B) Effect of high salt or detergent alone. Immunoprecipitates using mAb N3H10 or human autoimmune serum JM, specific for p70 plus p80, were washed with 1.5 M NaCl NET-NP40 (NaCl), MMB, or 1.5 M NaCl NET-NP40 followed by MMB (NaCl/MMB), then analyzed by SDS-PAGE and autoradiography. Positions of p70 and p80 are indicated.

As expected (Reeves, 1987; Chou et al., 1992), p70/p80 heterodimers purified from low salt extracts onto either 162 (Fig. 7A, left) or N3H10 (Fig. 7A, right) affinity beads bound radiolabeled *Hin*dIII fragments of bacteriophage lambda ranging from 23.6 to 0.12 kb. The binding was increased considerably by pretreating the beads with 1.5 M NaCl, which presumably elutes unlabeled cellular DNA from Ku (Reeves, 1987). When the beads were washed subsequently with MMB to dissociate p70 and p80, DNA binding was eliminated. This was the case for both 162 beads (which retain both p70 and p80 after high salt/MMB treatment) and N3H10 beads (which retain only p70, see Figs 5 and 6). However, when the beads were washed again with 1.5 M NaCl, DNA binding by both 162 and N3H10 beads was restored. Binding of the radiolabeled DNA (especially the 0.56 and 0.12 kb fragments) to N3H10-p70 beads was clearly visible, but was somewhat less efficient than binding to 162-p70 + p80 beads (compare Fig. 7, left and right panels, far right lane). Thus, cellular p70, like the recombinant human p70 protein (Chou et al., 1992), bound 'non-specifically' to linear double-stranded DNA fragments in vitro. MMB blocked the DNA-binding site either by binding directly to it, or by altering its conformation. Notably, the binding of DNA to p70 and p80 (162 beads) and to p70 alone (N3H10 beads) were both eliminated by MMB treatment, suggesting that DNA binding by p70 and p70/p80 dimers was similar in this respect. The non-uniform binding to different lambda DNA fragments (Fig. 7A and B) has been observed previously with p70 expressed in *E. coli* (Chou et al., 1992), but the explanation for this phenomenon is not known.

Similar results were obtained without high salt or detergent treatment using RK13 cells infected with p70-vacc, p80-vacc, or both viruses (Fig. 7B). As expected, the affinity-purified recombinant p70/p80 heterodimer bound end-labeled DNA fragments efficiently (Fig. 7B). A much weaker signal was also detected in mAb 162 immunoprecipitates of RK13 cells infected with wild-type (wt) vaccinia virus, probably due to binding of DNA to the endogenous rabbit Ku complex (see Fig. 4). Affinity-purified recombinant human p70 bound the labeled DNA fragments nearly as efficiently as p70/p80 heterodimers. In contrast, the binding of labeled DNA fragments to human p80, affinity-purified using mAb 111 from lysates of p80-vacc-infected RK13 cells, was not different from that of the controls (immunoprecipitates of uninfected RK13 cell lysate or wild-type vaccinia virus-infected RK13 cell lysate), suggesting that p80 may not bind DNA efficiently in the absence of p70. However, it should be noted that the quantity of p80 that could be purified from lysates of p80-vacc-infected RK13 cells was considerably less than the amount of p70 purified from p70-vacc-infected cells (Fig. 4).

Free p70 is associated with chromatin

Association of the recombinant Ku antigens in vivo with chromatin was examined by treating isolated nuclei with DNase and high salt. Previous studies suggest that a substantial portion of the nuclear Ku antigen can be released by DNase treatment (Reeves, 1985; Yaneva et al., 1985; Yaneva and Busch, 1986). In agreement with these previous observations, both p70 (Fig. 8, left panel) and p80 (not shown) were released efficiently from K562 by incubating nuclei for 30 minutes at 0°C in buffer containing 50 μg/ml DNase. Incubation of K562 cell nuclei with buffer alone released a smaller amount of p70/p80 heterodimer that was associated with DNA as shown by immunoprecipitation and agarose gel electrophoresis of bound nucleic acids (data not shown), consistent with its release by endogenous nucleases. The release of Ku from nuclei of p70-vacc/p80-vacc-coinfected cells by DNase was comparable to that from K562 cells (Fig. 8, right panel). Similarly, human p70 was released by DNase but not by buffer alone (Fig. 8, right panel), suggesting that the recombinant p70 protein was associated with chromatin in the nuclei of p70-vacc-infected RK13 cells. Release of endogenous rabbit p70 from cells infected with wild-type vaccinia virus was not detectable using mAb N3H10 (Fig. 8, right panel), probably due to the low level of Ku in RK13 cells. In contrast, little or no p80 was released by DNase from the nuclei of p80-vacc-infected cells; however, interpretation was complicated by the low level of p80 expression (data not shown). The release of human p70 from p70-vacc-infected cells by DNase treatment suggests that the p70 protein was associated with chromatin in vivo.

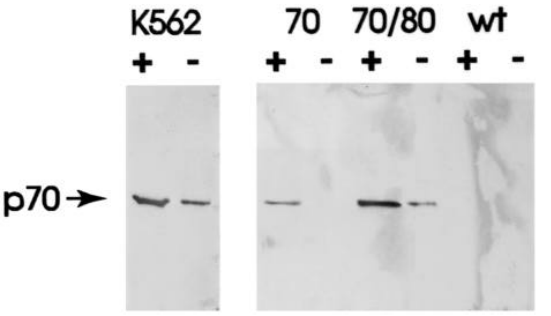
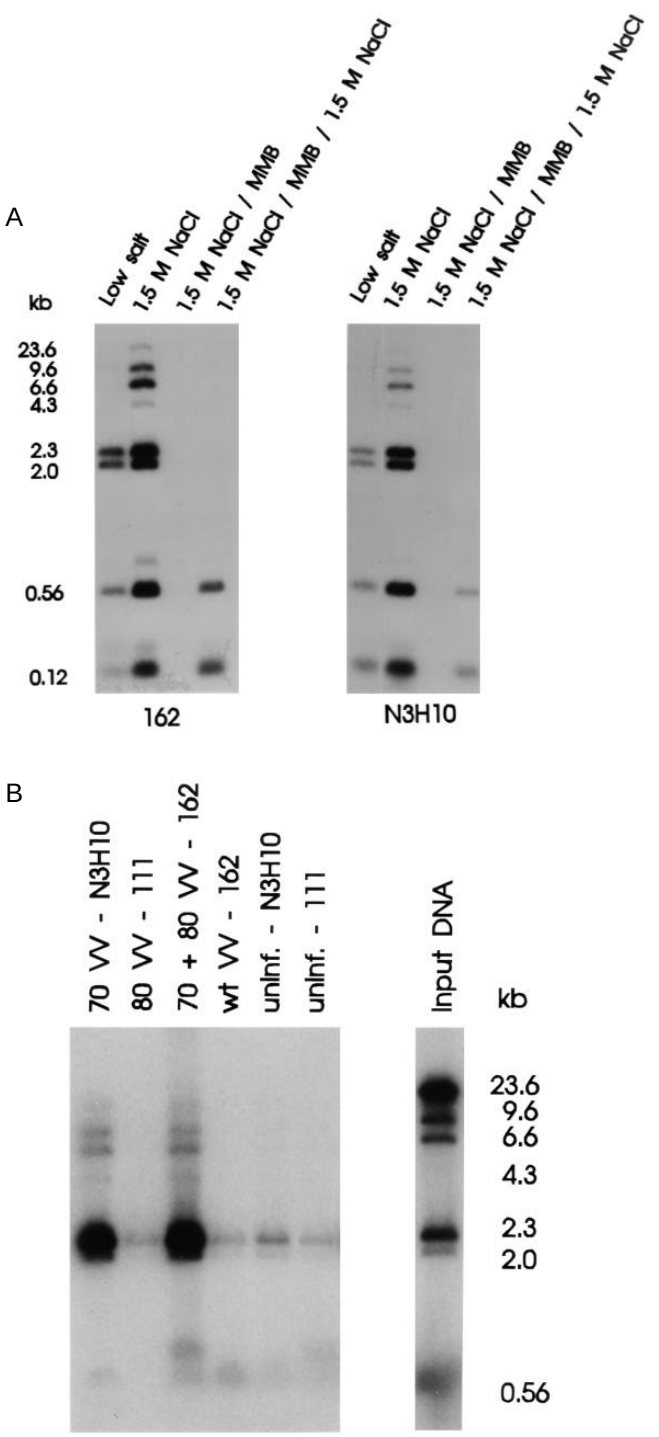


Fig. 8. Release of Ku from nuclei by DNase. Detergent nuclei isolated from uninfected K562 cells (left panel), or from RK13 cells infected with p70-vacc (70), p70-vacc plus p80-vacc (70/80) or wild-type (wt) vaccinia virus (right panel) were incubated for 30 minutes on ice in buffer containing 50 μg/ml DNase (+), or in buffer alone (–). Nuclei were removed by centrifugation, and released proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with mAb N3H10 (anti-p70). Position of p70 is indicated (arrow).



DISCUSSION

Although it has been established recently that Ku is the DNA-binding component of DNA-PK (Lees-Miller et al., 1990; Dvir

Fig. 7. Binding of DNA to free p70 in DNA immunoprecipitation assay. (A) Autoradiography of gel showing labeled DNA fragments isolated by binding to cellular p70. ^{32}P end-labeled λ*Hin*dIII fragments of 23.6-0.12 kb were allowed to bind to cellular Ku antigens affinity-purified on 162 (anti-p70/p80) or N3H10 (anti-p70) beads as described in Materials and Methods. Before adding radiolabeled DNA, the affinity-purified Ku antigens were washed with 0.15 M NaCl (Low salt), 1.5 M NaCl, 1.5 M NaCl followed by MMB, or sequentially with 1.5 M NaCl, MMB and 1.5 M NaCl. (B) Autoradiography of gel showing labeled DNA fragments isolated by binding to recombinant p70. ^{32}P end-labeled λ*Hin*dIII fragments were allowed to bind to affinity-purified Ku antigens from RK13 cells infected with p70-vacc (70 VV), p80-vacc (80 VV) or both viruses (70 + 80 VV). Human p70 was purified with mAb N3H10, p80 with mAb 111, and p70/p80 heterodimer with mAb 162. Immunoprecipitates from wild-type vaccinia virus (wt)-infected cells or uninfected RK13 cells are shown for comparison.

et al., 1992; Anderson and Lees-Miller, 1992; Gottlieb and Jackson, 1993), the precise roles of cellular Ku and DNA-PK in vivo are unclear, and the mechanism of DNA binding by Ku is controversial. The low level or complete absence of Ku antigen and DNA-PK activity in many non-primate cells (Bravo and Celis, 1982; Celis et al., 1987; Wang et al., 1993; Lees-Miller et al., 1992; Anderson and Lees-Miller, 1992) makes cell lines such as RK13 suitable for investigating the assembly and function of DNA-PK. mAbs, such as 162 and N9C1, which recognize intermediates in the assembly pathway of DNA-PK, will also facilitate these studies. In the present study, we have used 162 and other mAbs to examine the nuclear transport and DNA binding of free p70 and p80, and the assembly of p70/p80 heterodimers in rabbit kidney cells infected with recombinant vaccinia viruses directing the synthesis of human p70 and p80.

mAb 162 recognizes p70/p80 heterodimers

Although several mAbs that recognize Ku have been reported (Reeves, 1985; Wen and Yaneva, 1990; Knuth et al., 1990; Celis et al., 1987; Higashiura et al., 1992; Li and Yeh, 1992), 162 is the first shown to be specific for the p70/p80 heterodimer. The evidence that 162 recognizes the p70/p80 dimer is compelling. First, it is unreactive with cellular p70 and p80 or p70 or p80 fusion proteins on immunoblots, suggesting that it recognizes a conformational determinant (Wang et al., 1993). Second, 162 stained cells coinfected with p70-vacc plus p80-vacc, but not singly infected cells (Fig. 2), and immunoprecipitated p70 and p80 in approximately equal amounts from extracts of coinfected cells, but did not immunoprecipitate free p70 or p80 from extracts of singly infected cells (Fig. 4). Third, binding of 162 to Ku blocked the dissociation of p70/p80 heterodimers in vitro (Fig. 5), and finally, the antigen recognized by 162 migrated as a sharp 10 S peak on density gradients (data not shown), consistent with the mobility of p70/p80 heterodimers (Reeves, 1985). The specificity of 162 for p70/p80 heterodimers makes it useful for analyzing the molecular interactions of p70/p80 with p350 and DNA. Moreover, mAb 162 has proved valuable for examining the subcellular distribution of p70/p80 dimers and turnover of Ku (M. Satoh et al., unpublished data).

Nuclear transport and assembly of p70 and p80

It has been difficult in the past to assemble Ku heterodimers in active form, and the vaccinia system described here should make it possible to examine the functional effects of mutant Ku antigens in vivo, particularly when cDNAs encoding the p350 subunit become available. A more complete picture of the assembly, regulation and function of Ku and DNA-PK can be gained by combining immunofluorescence using specific mAbs with the expression of individual subunits in non-primate cells using recombinant vaccinia viruses. In the present studies, which were focused on validating the feasibility of this approach, we showed that the free p70 and p80 subunits undergo nuclear transport individually (Fig. 3). The nuclear transport of free human p70 and p80 is unlikely to be a consequence of vaccinia infection, since previous studies have demonstrated the appropriate nuclear transport or exclusion of proteins expressed by recombinant vaccinia viruses (Hong and Engler, 1991; Zhou et al., 1991; Nuesch and Tattersall, 1993). Nuclear transport is also unlikely to be related to dimerization with the small pool of endogenous rabbit Ku proteins, because the rabbit Ku proteins are 40- to 60-fold less abundant than human Ku subunits in the vaccinia-infected cells and in view of the lack of staining of cells that were singly infected with p70-vacc by mAb 162 (Fig. 2), and sucrose density gradient analysis indicating that much of the recombinant p70 and p80 in singly infected cells migrated at ~4 S rather than 10 S, consistent with the expected mobility of monomers (not shown). Taken together, our data suggest strongly that, unlike certain other nuclear proteins whose transport to the nucleus is facilitated by dimerization (Zhao and Padmanabhan, 1988; Booher et al., 1989), both p70 and p80 can be transported to the nucleus independently, an interpretation that is also corroborated by recent studies with uninfected human cells (M. Satoh et al., unpublished data). However, it remains to be determined if dimerization occurs in the cytoplasm or the nucleus, and whether chaperonins such as hsc-70 (Shi and Thomas, 1992) are involved in the nuclear transport of p70 and p80. Although both p70 (Reeves and Sthoeger, 1989) and p80 (Yaneva et al., 1989) contain sequences resembling nuclear localization motifs, further studies will be necessary to define their respective roles in facilitating nuclear transport.

Interaction of Ku with DNA

Like the cellular Ku antigen (Reeves, 1985; Yaneva and Busch, 1986; Yaneva et al., 1985), human p70/p80 dimers assembled in RK13 cells coinfected with p70-vacc plus p80-vacc were released from nuclei by DNase treatment (Fig. 8), and bound to DNA in vitro (Fig. 7B), suggesting an association with chromatin. Similarly, both DNase release and in vitro DNA-binding assays suggested that recombinant free p70 was also associated with chromatin in vivo, consistent with previous in vitro studies (Chou et al., 1992). Moreover, the binding of both cellular and recombinant free p70 to DNA was quantitatively similar to that of p70/p80 heterodimers (Fig. 7A and B), and DNA binding by both the free p70 protein and p70/p80 dimers was reversibly eliminated by MMB treatment (Fig. 7A). It should be mentioned that the DNA binding by both forms (free p70 and p70/p80 dimer) after MMB/high salt treatment may differ somewhat from that of the untreated Ku antigen (Fig. 7). The preferential binding of smaller DNA fragments after MMB/high salt may be related to incomplete refolding of Ku after exposure to the detergent, as suggested by previous observations that a prolonged renaturation step facilitates DNA binding to the larger lambda DNA fragments in southwestern blots (Chou et al., 1992). It is unlikely that DNA binding is a non-specific effect of MMB treatment on proteins in general, because affinity beads coated with mAbs in the absence of Ku failed to bind DNA after MMB/high salt washing (data not shown; and J. Wang et al., unpublished data). However, the explanation for the non-uniform binding of Ku to lambda DNA fragments after MMB/high salt washing remains incompletely understood, and further studies of this phenomenon will be needed.

Detailed DNA binding studies completed recently indicated that free p70, like the p70/p80 dimer, displays preferential binding to the termini of double-stranded DNA, further supporting the idea that free p70 is the DNA-binding component of Ku (J. Wang et al., unpublished data). This appears not to be related to stabilization of the structure of p70 as a consequence of mAb binding because gst-p70 fusion proteins

purified on glutathione-agarose beads bind DNA comparably to the same fusion proteins purified using mAbs (J Wang et al., unpublished data). Moreover, the binding of free p70 versus p70/p80 heterodimers to DNA showed similar sensitivity to detergent and salt washes. The nuclear transport of free p70 and p80 (Figs 2 and 3) suggests that free p70 might gain access to and bind chromatin before dimerizing with p80, and we have recently identified a free form of p70 in uninfected human K562 cells (M. Satoh et al., unpublished data). However, in vitro translated p70 does not bind as efficiently as p70/p80 heterodimers to ^{32}P-labeled DNA in gel shift assays in the presence of high concentrations of poly(dI-dC), whereas the p70/p80 dimer does (A. Pierani, unpublished data), suggesting that subtle differences in DNA-binding preference or affinity may exist. A qualitative or quantitative difference in the binding of p70/p80 dimers versus p70 monomers to DNA would not be unexpected, in view of a recent report that p70 translated in vitro does not bind DNA, whereas p70/p80 dimers bind efficiently (Griffith et al., 1992). Nevertheless, the present studies raise the possibility that the DNA-PK complex assembles on chromatin. We have shown that newly synthesized p70 gains access to the nucleus and becomes associated with chromatin, at least in the vaccinia system, and more recent data suggest that free p70 and the p70/p80 dimer display similar DNA-binding preferences (J. Wang et al., unpublished data). These observations may suggest that newly synthesized p70 binds to DNA first, followed by the binding of p80 and p350. The nuclear transport and DNA binding of newly synthesized free p70 might play a role in regulating DNA-PK function. Additional studies using specific mAbs and recombinant vaccinia viruses directing the synthesis of individual DNA-PK subunits may help to define further the biological role of the Ku autoantigen.

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